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## A rapid and simple fluorescence enzyme-linked immunosorbent assay for tetrabromobisphenol A in soil samples based on a bifunctional fusion protein

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### Abstract

Tetrabromobisphenol A (TBBPA) is the largest brominated flame retardant which can be released to environment and cause long-term hazard. In this work, we developed a rapid and highly sensitive fluorescence enzyme-linked immunosorbent assay (FELISA) for monitoring of TBBPA in soil samples. TBBPA specific nanobody derived from camelid was fused with alkaline phosphatase to obtain the bi-functional fusion protein, which enable the specific binding of TBBPA and the generation of detection signal simultaneously. The assay showed an IC<sub>50</sub> of 0.23 ng g<sup>-1</sup>, limit detection of 0.05 ng g<sup>-1</sup> and linear range from 0.1 to 0.55 ng g<sup>-1</sup> for TBBPA in soil samples. Due to the high resistance to organic solvents of the fusion protein, a simple pre-treatment by using 40% dimethyl sulfoxide (DMSO) as extract solvent can eliminate matrix effect and obtain good recoveries (ranging from 93.4% to 112.4%) for spiked soil samples. Good relationship between the results of the proposed FELISA and that of liquid chromatography tandem mass spectrometry (LC-MS/MS) was obtained, which indicated it could be a powerful analytical tool for determination of TBBPA to monitor human and environmental exposure.

### Keywords

Tetrabromobisphenol A; Nanobody; Fluorescent immunoassay; Soil detection; Alkaline phosphatase

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Conflict of interest

The authors declare no conflict of interest.

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## 1. Introduction

Tetrabromobisphenol A (TBBPA) with high global production (over 200000 t a year) (Honkisz and Wojtowicz, 2015) is the most ubiquitous brominated flame retardants (BFRs) in industry and commerce to reduce fire-related injury. However, as an additive flame retardant, TBBPA often is simply blended with polymers and more easily leached out of products into the environment during production, usage and disposal. There is evidence of association with particles and bioaccumulation (Howard and Muir, 2010; Liu et al., 2016). TBBPA is now detectable in environmental media such as soil (Lu et al., 2018; Sun et al., 2014), dust (Barghi et al., 2017), sediment (Cheng and Hua, 2018), water (Chokwe et al., 2017), aquatic organisms (Ashizuka et al., 2008; de Jourdan et al., 2013), human being blood (Lu et al., 2017) and breast milk (Antignac et al., 2009; Shi et al., 2009). Highly industrialized countries were observed relatively high TBBPA levels in environmental monitoring such as China, Germany, Japan, Korea, the UK and the USA (Abdallah et al., 2008; Fromme et al., 2014; Wang et al., 2015). Even though the threshold reference value of TBBPA suggested by the US Environmental Protection Agency is  $1 \times 10^6$  ng kg<sup>-1</sup> of bw day<sup>-1</sup>. More and more studies indicated that TBBPA exposure might induce various detrimental effects to mammals, such as neurotoxicity (Alzualde et al., 2018; Cannon et al., 2019), hepatotoxicity (Parsonsa et al., 2019) and reproductive toxicity (Linhartova et al., 2015; Zhang et al., 2018). People are exposed to TBBPA daily. The potential toxicity of TBBPA has led to concern for public health. Therefore, it is essential to establish approaches to monitor TBBPA exposure.

For reducing the risks of TBBPA exposure to consumers, great efforts have been expended on TBBPA detection. Among the numerous methods, immunoassays due to their advantageous properties such as high throughput, simplicity and straightforward readouts have been widely applied for ordinary analysis for large numbers of samples. In the construction of immunoassays, the single variable domains of heavy chain (VHH) antibodies derived from camelids and sharks, also named as nanobodies, offer advantages with a small size of 15000 molecular weight, low cost, high solubility, stability and high expression in microbial systems, make nanobody an alternative to conventional antibodies (monoclonal antibodies, polyclonal antibodies and single-chain variable fragments) (Bever et al., 2016). With the rapid evolution of antibody engineering and the gene recombinant techniques, antibodies being fused with enzymes as homogeneous probes are used instead of the secondary antibodies with chemically-coupled enzymes like horseradish peroxidase (HRP) and alkaline phosphatase (AP). Furthermore, many published studies confirmed that enzyme linked immunosorbent assays (ELISA) which depend on fluorescence as signal output can improve the sensitivity of assays compared with the routine absorbance (Huo et al., 2018; Lassabe et al., 2018; Zhang et al., 2018). However, a few fluorescence assays have been reported for small molecules detection with VHH-AP fusion protein currently.

Herein, based on previously prepared anti-TBBPA VHH (Wang et al., 2014), we clone the VHH gene into the expression vector pLIP6/GN, which contained the AP gene to produce the anti-TBBPA VHH-AP fusion protein. The VHH can specific bind TBBPA in samples while the AP is able to catalyze 2'-[2-benzothiazoyl]-6'-hydroxybenzothiazole phosphate (BBTP) to generate fluorescence. Therefore, one-step fluorescence enzyme-linked

immunosorbent assay (FELISA) was developed and applied to determine TBBPA in soil samples. The assay conditions were well optimized to obtain optimum performance. Recoveries test was performed and the results were validated with standard LC-MS/MS.

## 2. Materials and methods

### 2.1. Chemicals and Reagents

The synthesis of haptens of TBBPA and production of VHH against TBBPA were described in previous report (Wang et al., 2014). The pLIP6/GN vector containing AP gene used was a gift from Dr. Frédéric Ducancel from Pharmacology and Immunoanalysis Department, CEA/Saclay, Gif-sur-Yvette, France. The restriction enzymes *Sfi*I and *Not*I and T4 DNA ligase were obtained from Thermo Fisher Scientific (Thermo, USA). The chemically competent cells of *E. coli* BL21 (DE3) pLysS and His-tag antibody-HRP were from Transgene Biotech (Guangzhou, China) was used for expression of the antibody fusion protein. The fluorescent substrate (2'-[2-benzothiazoyl]-6'-hydroxybenzothiazole phosphate, BBTP) was obtained from Promega (WI, USA). Standards were purchased from TCI Co. Ltd. (Tokyo, Japan) and other TBBPA analogues were purchased from AccuStandard (New Haven, CT). All other reagents were of analytical grade and purchased from Qixiang Technology Co., Ltd. (Guangzhou, China).

### 2.2. Construction of the recombinant plasmid

VHH genes of TBBPA with complementary *Sfi*I and *Not*I restriction sites were amplified by PCR (forward primer: 5'-ATATGGCCCAGCCGGCCACCATCACCATCACCATCAGGTGCAGCTCGTGG AG-3'; reverse primer: 5'-ATAAGAATGCGGCCGCGTCTTGTGGTTTTGGTGTCTTG-3'). The purified PCR products after digesting with *Sfi*I and *Not*I were purified again and subcloned into the pLIP6/GN using T4 DNA ligase. Then, the chemically competent cells BL21 (DE3) pLysS were transformed with recombinant anti TBBPA VHH-AP plasmid by heat shock at 42 °C for 90 s and grown on LB plates overnight at 37 °C. The positive clones were selected for sequence identification.

### 2.3. Expression and purification of the anti-TBBPA VHH-AP fusion protein

The positive clone with the correct sequence was induced to express anti-TBBPA VHH-AP fusion protein, which was cultured in SB medium with 100 µg mL<sup>-1</sup> ampicillin at 37 °C until the OD<sub>600</sub> reached the value of approximately 0.4–0.8. Then 1 mmol L<sup>-1</sup> of IPTG was added to induce protein expression. After incubation overnight at 37 °C with shaking at 250 rpm, bacteria were pelleted by centrifugation at 13523 g for 20 min at 4 °C. The fusion protein was extracted from cell periplasmic using cold osmotic shock method (Olichon et al., 2007) and then purified with Ni-NTA resin by using 200 mmol L<sup>-1</sup> imidazole in 0.01 mol L<sup>-1</sup> PBS for elution and stored at -20 °C after dialysis against 0.01 mol L<sup>-1</sup> PBS. The 12% SDS-PAGE and western blotting were both applied to identify the resulting anti-TBBPA VHH-AP fusion protein.

#### 2.4. One-step FELISA performance

The anti-TBBPA VHH-AP fusion protein was characterized by FELISA, which was performed on the 96-well black opaque microplates (Scheme 1). First, the coating antigen (shown in Fig. S1) was diluted with the carbonate buffer (100  $\mu\text{L}$  per well) and incubated overnight at 37  $^{\circ}\text{C}$ . After washing twice with PBST (200  $\mu\text{L}$  per well). Blocking solution (120  $\mu\text{L}$  per well, 0.01mol  $\text{L}^{-1}$  PBS with 2% skimmed milk) was added to each well for 3 h at 37  $^{\circ}\text{C}$ . The 50  $\mu\text{L}$  TBBPA standard solutions and the diluted VHH-AP fusion protein (50  $\mu\text{L}$ ) in PBST were pipetted into the wells of the plates. After 30 min of incubation, the plates were washed five times again with PBST. And then 100  $\mu\text{L}$  of AP fluorescent substrate BBTP was added and incubated at 37  $^{\circ}\text{C}$  for 15 min. The fluorescence intensities of the emission wavelength at 555 nm were measured under the excitation wavelength of 435 nm. We utilized the inhibition to characterize the binding ability of fusion protein. (Inhibition =  $[1 - (F/F_0)] \times 100$ , where  $F_0$  and  $F$  were defined as the fluorescence in absence and in the presence of TBBPA, respectively).

#### 2.5. Optimization of FELISA conditions

To achieve highly sensitive detection for TBBPA, several experimental parameters which could affect the FELISA performance were optimized. There involved the concentration of anti-TBBPA VHH-AP fusion protein and coating antigen, the effects of organic solvents (5%, 10%, 20%, 40% and 60% of MeOH, DMSO) and the value of pH (4–11). For each optimized condition, the inhibition curves ( $n=3$ ) with TBBPA as competitor analyte were established. The value of the concentration of the TBBPA leading 50% inhibition ( $\text{IC}_{50}$ ) was the primary criteria for evaluating assay performance. Furthermore, the lower  $\text{IC}_{50}$  equates to the higher sensitivity of the assay. In order to achieve short time-consuming of assay procedure, we also explored shortened incubation times of fusion protein and antigen.

#### 2.6. Sample preparation and analysis

TBBPA ( $\log k_{\text{ow}}$  3.2–6.4) was used as flame retardant in plastics and can be easily released to the environment and distribute into soil, during their production and usage processes. Considering this, the surface soil (0–5 cm deep) sample near the plastics factory was selected for TBBPA pollutants detection. Blank soil samples were evaluated by liquid chromatography tandem mass spectrometry (LC-MS/MS) to ensure TBBPA-free, which was used as a negative control. For soil sample preparation, initially, the samples were heated at 50  $^{\circ}\text{C}$  for 12 h, and then sieved through 50-mesh screen. A dry weight (dw) of 1.0 g soil was spiked with a range concentration of TBBPA (52.4, 65.5, 81.9, 102, 128, 160 and 200  $\text{ng g}^{-1}$ ) and added 2.5 mL of 40% DMSO in 0.01mol  $\text{L}^{-1}$  PBS for the extraction. After sonication for 30 min and the tube was centrifuged at 8000 g for 10 min and the supernatant was collected. The extracts were filtered through a 0.22  $\mu\text{m}$  filter and then used for analysis (when needed the extract can be diluted with 40% DMSO in 0.01mol  $\text{L}^{-1}$  PBS). The LC-MS/MS was applied to verify the accuracy and reliability of the proposed FELISA. As a reference method in accordance with the Chinese national standards, chromatography was achieved using gradient elution with the mixture of the 20 mmol  $\text{L}^{-1}$  ammonium acetate (A) and acetonitrile (B): 0–3 min, 10% B; 3–6 min, 10% to 90% B; 6–15min 10% B. The injection volume was 5  $\mu\text{L}$  with the flow rate 0.35  $\text{mL min}^{-1}$ . Analytes were determined by

ESI-MS/MS in the negative mode. Other parameters were as follows: Heat block temperature: 450 °C; Drying gas flow: 15 L min<sup>-1</sup>; Nebulizer gas flow: 3 L min<sup>-1</sup>; Detection voltage: 2.22 kV; DL temperature: 280 °C, CID gas, 230 kPa. The parent ion and the daughter ion of TBBPA was m/z 542.70 and m/z 446.00, respectively, which were used for quantitation. The calibration curves ranged from 20 and 400 ng mL<sup>-1</sup> (R<sup>2</sup>=0.991). Limit of quantification was 1.2 ng mL<sup>-1</sup> (S/N=3).

### 3. Results and discussion

#### 3.1. Expression, purification, and identification of the anti-TBBPA VHH-AP fusion protein

According to the description in the experimental section, the VHH gene was inserted into the expression vector pLIP6/GN to express the anti-TBBPA VHH-AP fusion protein. The positive recombinant plasmid containing the gene of the anti-TBBPA VHH-AP fusion protein was obtained and confirmed by DNA sequencing (amino acid sequences were shown in Fig. S2). Then the recombinant plasmid was transformed into chemically competent cells (*E. coli* BL21 (DE3) pLysS). Fig. 1 showed that the anti-TBBPA VHH-AP fusion were expressed and extracted efficiently with sucrose osmotic pressure method and can be purified by using the Ni-NTA affinity chromatography, which existed in a sharp and clear protein band on SDS-PAGE gel and western blotting. The collected pure the anti-TBBPA VHH-AP fusion protein with an initial concentration of ~15 mg L<sup>-1</sup> that completely satisfied follow-up application. Moreover, the pLIP6/GN we applied has presents two mutations at the catalytic site. As a result, the catalytic activity of the alkaline phosphatase produced by this system was improved 35-fold than that of wild-type alkaline phosphatase (Dong et al., 2012; Muller et al., 2001).

#### 3.2. Optimization of the FELISA

Generally, the performance of the immunoassay is significantly influenced by the assay conditions. In our work, initially, the checkerboard titration method was applied to screen the optimal working concentrations of the the coating antigen and the VHH-AP fusion protein. As shown in (Table S1), the highest inhibition (TBBPA, 1 ng mL<sup>-1</sup>) was achieved when the concentration of coating antigen and fusion protein were at 100 ng mL<sup>-1</sup> and 0.14 µg mL<sup>-1</sup>, respectively. For suitable buffer optimization, because the TBBPA is a highly lipophilic analytes, which should be solubilized completely in the organic solvents such as dimethyl sulfoxide (DMSO) and (methanol) MeOH. Besides, it was reported that single domain antibodies often show an excellent resistance in organic solvents (Liu et al., 2014; Zhang et al., 2018). Considering these two issues, serial concentrations of TBBPA standards prepared by varied MeOH (Fig. 2A)/DMSO (Fig. 2B) concentrations (0, 5%, 10%, 20%, 40% and 60%) were used to construct FELISA inhibition curves for TBBPA. Fig. 2B shows that the lowest IC<sub>50</sub> (0.09 ng mL<sup>-1</sup>) observed at 0.01 mol L<sup>-1</sup> PBS with 40% DMSO, so the 0.01 mol L<sup>-1</sup> PBS containing 40% DMSO was chosen assay buffer for further studies. According to the previous report (Huo et al., 2018), the value of pH is also an important factor for immunoreactions. Different pH values (pH 4.0–11.0) were evaluated (see Fig. 2C, D). In test, optimal time was observed 6 and 8 with sensitivity decreasing outside at this range. Hence, the best performance was obtained in 0.01 mol L<sup>-1</sup> PBS (40% DMSO, pH 7.0). Furthermore, as Fig. 2E shown, a 30 min incubation time was enough for binding

reaction of VHH-AP fusion protein and antigen, which indicated that based on the anti-TBBPA VHH-AP fusion protein, the assay time is extremely shortened compared with classic ELISA.

### 3.3. Sensitivity and specificity of the FELISA

Considering the overall data, the TBBPA standard curve (Fig. 2F) in working buffer was constructed following the same procedure as described above. The concentration of anti-TBBPA VHH fusion protein binding to TBBPA causing half-maximum inhibition ( $IC_{50}$ ) was  $0.09 \text{ ng mL}^{-1}$ , and the limit of detection (LOD, 10% inhibited binding,  $IC_{10}$ ) was  $0.02 \text{ ng mL}^{-1}$ , and the linear working range ( $IC_{20} \sim IC_{80}$ ) from  $0.04$  to  $0.22 \text{ ng mL}^{-1}$  ( $y = -0.731571x - 0.31592$ ,  $R^2 = 0.989$ ), which were all calculated by the formula of FELISA standard curve.

Cross reactivity experiments were conducted to determine the specificity of the FELISA for TBBPA compared to the structurally related TBBPA congeners. Therefore, 2, 2', 6, 6'-tetrabromobisphenol A diallyl ether, bisphenol A, BDE-47, BDE-99, tetrabromobisphenol A bis(2-hydroxyethyl) ether and hexabromocyclododecane were selected as inhibitors under the same experimental conditions. Additionally, the CRs of anti-TBBPA VHH fusion protein for TBBPA and its analogues were evaluated on the basis of  $IC_{50}$  with following formula:  $CR(\%) = IC_{50}(\text{TBBPA, ng mL}^{-1}) / IC_{50}(\text{TBBPA analogues, ng mL}^{-1}) \times 100\%$ . The results (Fig. 3) revealed that the negligible CR ( $< 0.1$ ) was observed with all the other analogues which suggesting that VHH-AP-fusion protein based FELISA is highly selective for TBBPA. The acceptable specificity of this assay makes it valuable for broad application.

### 3.4. Stability of anti-TBBPA VHH-AP fusion protein

The thermal stability study of anti-TBBPA VHH-AP fusion protein was investigated by simultaneously testing the effects of different temperature on the binding activities with coating antigen. As shown in Fig. S3A, the binding activities of the VHH-AP fusion protein was decreased as temperature increased from  $37$  to  $87 \text{ }^\circ\text{C}$ . And the fusion protein retained around  $37.5\%$  binding activity after incubating at  $67 \text{ }^\circ\text{C}$  for  $40 \text{ min}$ . The result demonstrated the anti-TBBPA VHH fused the AP showed poorer thermostable than VHH of TBBPA studied before (Wang et al., 2014), which is may be influenced by the thermal denaturation of AP and the irreversible refolding process (Liu et al., 2013). Apart from thermo stability, the stability at  $25 \text{ }^\circ\text{C}$  was also measured. In terms of result (Fig. S3B), the VHH-AP-fusion protein exhibited superior stability in  $25 \text{ }^\circ\text{C}$  for  $35 \text{ days}$ . The expected results illustrated that the AP fusion protein only made little influence on the native activity of antibody, when fused with a single domain antibody, which might be due to antibody protein maintaining its original refolding way.

### 3.5. Sample analysis and validation

The matrix effect is a crucial factor that must not be ignored which often determines the accuracy and sensitivity of immunoassays for sample analysis. TBBPA is soluble in organic reagents. Therefore, an organic solvent is necessary to apply especially in the sample extraction progress. Consequently, the representative sample were spiked with different concentrations ( $52.4, 65.5, 81.9, 102, 128, 160$  and  $200 \text{ ng g}^{-1}$ ) of TBBPA and extracted as

described above and analyzed in triplicate. As expected, no significant matrix interference was observed, when the extracting solution was 2.5-fold diluted. The calibration curve for sample (Fig. S4) is consistent with that in 0.01 mol L<sup>-1</sup> PBS containing 40% DMSO. Furthermore, the average recoveries of TBBPA via the FELISA for the samples spiked were in a range of 93.4%–112.4% and the coefficient of variance (CV) ranged from 1.6% to 8.1% (see Table 1). For evaluating the accuracy and the precision of FELISA, the LC-MS/MS was applied to test same samples. Meanwhile, the acceptable recoveries of the LC-MS/MS from 100.2% to 110.0% with the coefficient of variation of 4.0%–7.4% (Table 1) were obtained. As shown in Table 1, the *t* test results showed that the P values of each spiked levels were above 0.05, indicating that no significant difference were encountered between the two methods. The results also indicated that simple ultrasonic extraction and dilution with DMSO is a reasonable pretreatment approach for TBBPA determination in soil via the FELISA, which exhibited a good correlation with the result of LC-MS/MS ( $R^2=0.95$ , Fig. 4). In addition, to further assess the applicability of proposed FELISA with this fusion protein, we measured the soil samples from the six different companies which produced plastic materials in Guangzhou, China and analyzed them by the FELISA and LC-MS/MS in a blind fashion. As presented in Table 2, No.4 sample showed strong positive by the proposed FELISA (not quantitative due to exceed of upper quantitative limit), which was detected containing 176 ng mL<sup>-1</sup> by LC-MS/MS. The detection results obtained from the FELISA and LC-MS/MS were in good agreement with each other, which further indicated the developed FELISA based on anti-TBBPA VHH-AP fusion proteins could become an effective tool for TBBPA analysis in soil samples.

#### 4. Conclusions

In conclusion, we successfully demonstrated a new type and reliable FELISA based on the anti-TBBPA VHH-AP fusion protein for screening TBBPA in soil samples. Compared to conventional ELISA applying monoclonal antibodies, FELISA is a more economical strategy, which can be attributed to the fact that fusion protein retained both specificity and affinity of VHH, catalytic activity of alkaline phosphatase and was less expensive to prepare. It could also be easily expressed in *E.coli* using simple genetic approaches resulting high yield. Moreover, because the alkaline phosphatase was directly linked to the anti-TBBPA VHH rather than by a chemical labeling method. The inactivation or activity decrease of antibody was avoided. Furthermore, FELISA avoids the number steps like secondary antibody incubating and relevant washing, which resulted in the assay procedure time reducing. For actual soil samples detection, the results of proposed method shows good correlation with that in LC-MS/MS. Overall, the FELISA based on the VHH-AP fusion protein can serve as an ultra-sensitive, simple, accurate and rapid analytical tool for monitoring the TBBPA in soil, which provide an alternative strategy for other lipophilic small molecule contaminants analysis.

#### Supplementary Material

Refer to Web version on PubMed Central for supplementary material.



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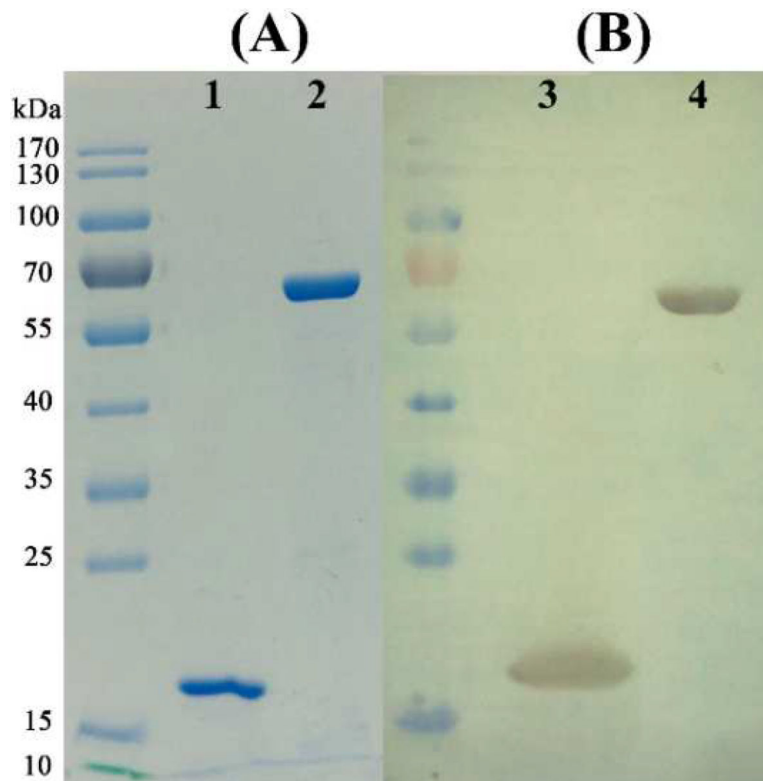
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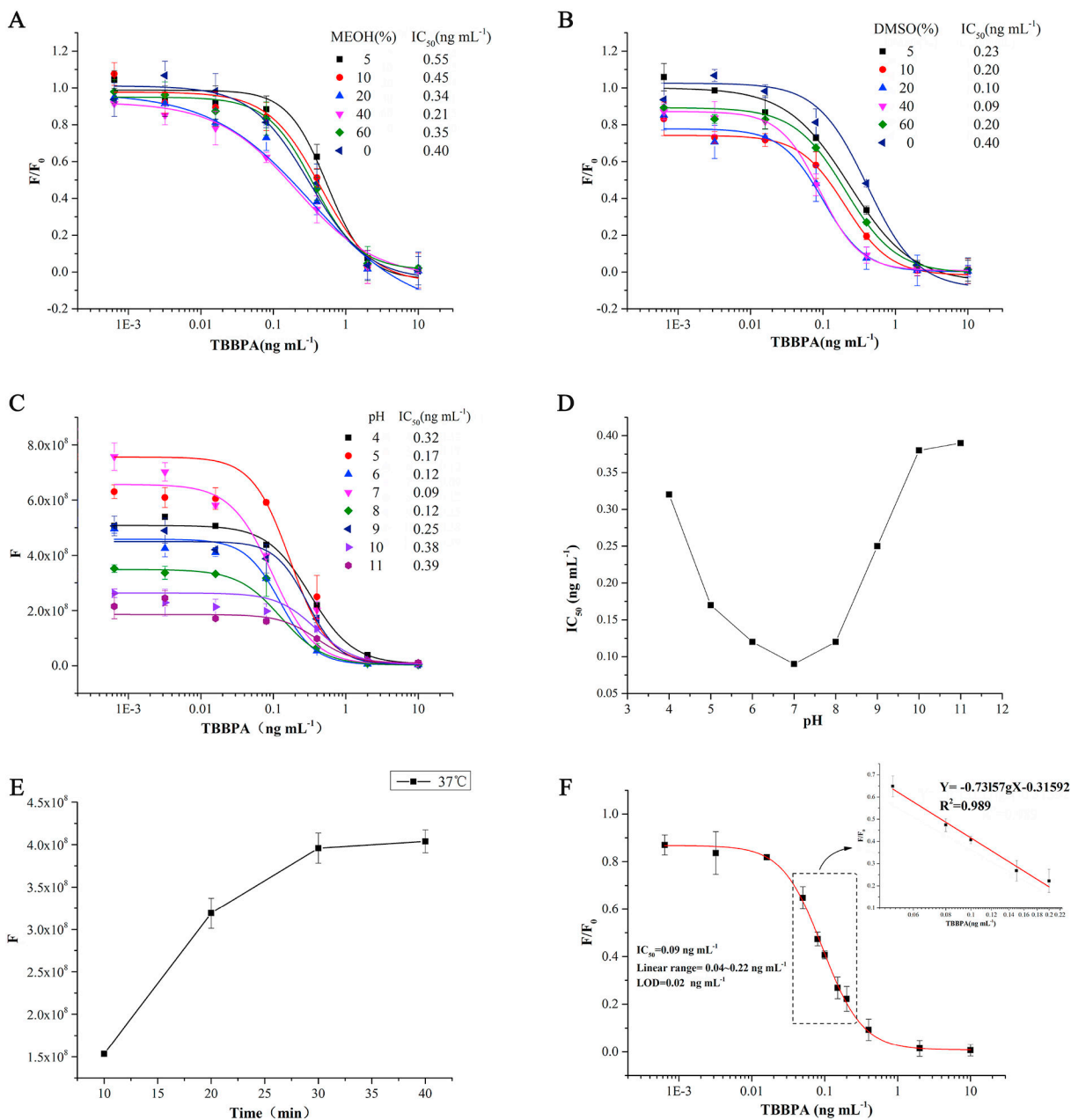
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### Highlights

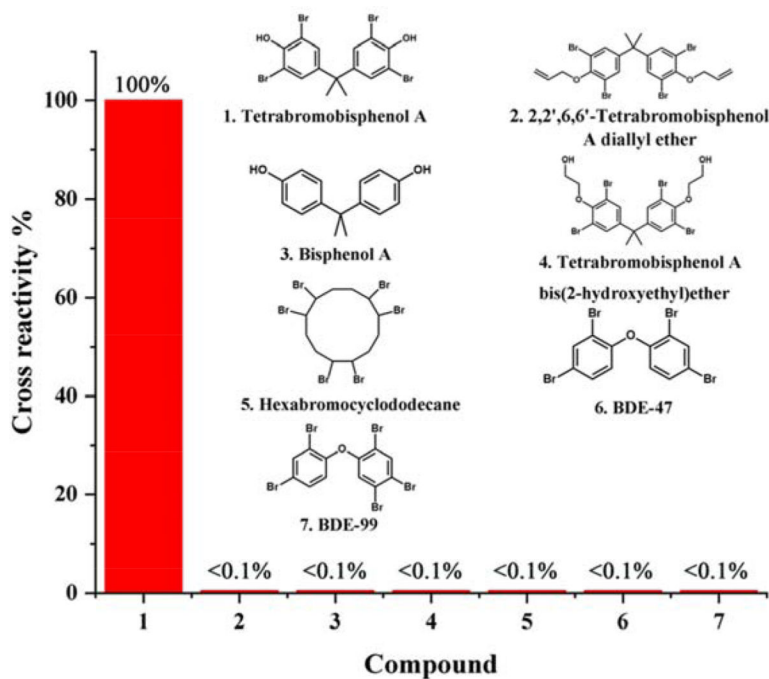
- A bifunctional nanobody-alkaline phosphatase fusion protein for TBBPA was produced.
- One-step fluorescence enzyme-linked immunosorbent assay for TBBPA was developed.
- The assay can determine TBBPA with highly sensitivity and simple pretreatment.
- The results were validated by standard LC-MS and showed good accuracy.



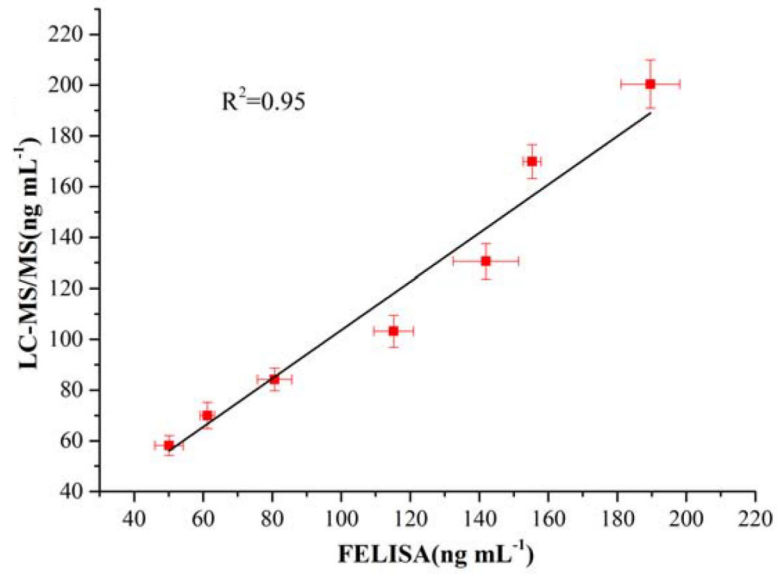
**Fig. 1.** Characterization of anti-TBBPA VHH-AP fusion protein by SDS-PAGE and Western blotting analysis based on anti-His antibody. (A) SDS-PAGE lane 1, purified anti-TBBPA VHH, lane 2, purified anti-TBBPA VHH-AP fusion protein. (B) Western blotting lane 1, purified anti-TBBPA VHH; lane 2, purified anti-TBBPA VHH-AP fusion protein.



**Fig. 2.** Effects of MeOH (A), DMSO (B), pH (C, D), and different incubation time on the performance of VHH-AP fusion protein based FELISA (E); Standard competitive inhibition curve (F) for TBBPA analysis under the optimized conditions. The error bars represent the standard deviation (n = 3).

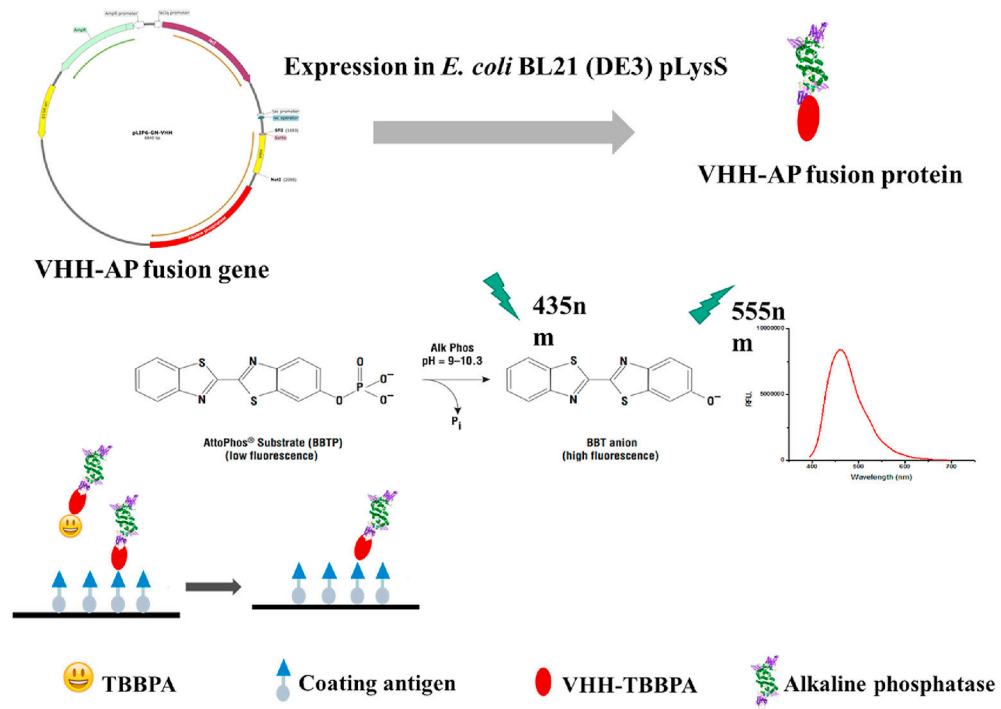


**Fig. 3.** Cross-reactivity of related analogues in the anti-TBBPA VHH-AP fusion protein based FELISA. (The percentage of CR was calculated using the following equation:  $CR (\%) = [IC_{50} (TBBPA, \text{ng mL}^{-1}) / IC_{50} (\text{cross-reactant, ng mL}^{-1})] * 100.$ )



**Fig. 4.** Correlations of analysis of samples spiked with TBBPA between FELISA and LC-MS/MS (n=3).



**Scheme 1.**

Schematic illustration of the anti-TBBPA VHH-AP fusion protein based FELISA method for quantitative detection of TBBPA.

**Table 1**

Results of spiked soil sample analyzed by FELISA and LC-MS/MS (n =3).

TBBPA spiked (ng/mL)	FELISA (Mean±SD <sup>b</sup> , ng/mL)	Recovery (%)	CV <sup>c</sup> (%)	LC-MS/MS (Mean±SD, ng/mL)	Recovery (%)	CV <sup>c</sup> (%)	P value
0	ND <sup>a</sup>	ND	ND	ND	ND	ND	ND
52.4	50.1±4.1	95.6	8.1	58.2±3.9	110.0	6.7	0.068
65.5	61.2±2.1	93.4	3.4	70.0±5.2	106.8	7.4	0.073
81.9	80.7±5.0	98.5	6.2	84.2±4.5	102.8	5.3	0.420
102	115.2±5.7	112.4	4.9	103.2±6.3	101.1	6.1	0.071
128	141.9±9.5	110.8	6.7	130.6±6.9	102.0	5.2	0.171
160	155.3±2.6	93.9	1.6	169.9±6.7	106.2	4.0	0.065
200	189.6±8.5	94.8	4.4	200.4±9.5	100.2	4.7	0.109

<sup>a</sup>ND = Not detectable<sup>b</sup>SD=Standard deviation<sup>c</sup>CV= Coefficient of variance.

**Table 2.**

Comparison of the blind analysis results for TBBPA content in soil samples from the six different companies by FELISA and LC-MS/MS (n=3).

Sample	FELISA (ng mL <sup>-1</sup> ) Mean ± SD <sup>a</sup>	LC-MS/MS (ng mL <sup>-1</sup> ) Mean ± SD
NO.1	ND <sup>b</sup>	ND
NO.2	ND	ND
NO.3	ND	ND
NO.4	Positive <sup>c</sup>	176.7±4.5
NO.5	ND	ND
NO.6	ND	ND

<sup>a</sup>SD, standard deviation

<sup>b</sup>ND, not detectable

<sup>c</sup>Positive, signal value exceeds upper quantification limit.

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